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In Vitro Assay of Cytotoxicity with Cultured Liver: Accomplishments and Possibilities

by Joe W. Grisham,* Ronald K. Charlton,* and David G. Kaufman*

Tissue cultures offer potential advantages for assaying the toxicity of chemicals and for evaluating tissue susceptibility to toxic agents. Several properties of cultured cells hinder the immediate, widespread use of tissue cultures to assay toxicity routinely. These points are illustrated by briefly reviewing attempts to utilize different types of hepatic cultures to evaluate the actions of carcinogenic chemicals *in vitro*. Hepatocytes *in vivo* apparently can metabolize all known procarcinogenic chemicals, but the process of tissue isolation and the environmental conditions *in vitro* may modify drastically the responses of hepatocytes and other cultured hepatic cells to toxic chemicals. Before cell cultures can be used routinely as the basis of screening systems to detect chemical toxins, specificity and sensitivity of response to chemicals representing all chemical classes must be validated by laboratory studies.

Introduction

The use of tissue cultures to assess the toxicity of chemicals has appealed to many scientists, although this technique is not yet widely used. As indicated by the attitude expressed recently in a publication of the World Health Organization (*1*), some toxicologists are loath to extrapolate to intact animals the results of cytotoxicity evaluations on cells cultured *in vitro*. This meeting to define positions concerning the application of tissue cultures to toxicity evaluation is timely. From the deliberations of this forum may come reasoned policies to guide the utilization of tissue cultures to assay the toxic potential of chemicals.

Despite the reticence of some workers, tissue cultures hold several potential advantages over laboratory animals as a means to evaluate the cellular effects of chemicals (*2, 3*). A most appealing attribute of cell culture is that cells from the species and the organ or tissue at risk may be utilized, perhaps allowing cellular aspects of species- and organ-specific toxicity to be studied. A major disadvantage of most cell culture methods available until recently is that metabolically limited, fibroblastoid cells have been used, allowing only nonspecific toxicity of eukaryotic cells to directly active chemicals to be studied. Recent ability to culture a variety of functionally competent epithelial cells, at least for short periods, improves the conditions for evaluating the toxic potential of a wide variety of chemicals, including protoxins, and it makes it possible to study epitheliocyte-specific toxicity *in vitro*. Epithelial cells are better able to activate protoxic chemicals metabolically than are mesenchymal cells (*4*), a fact that may correlate with the greater susceptibility of epithelium to toxic chemicals *in vivo*. Other advantages of tissue culture methods are their relative cheapness compared to animal studies; their freedom from organismic influences, such as blood flow and hormonal factors; and their facility to provide insight into cellular mechanisms that underlie toxicity. Essential requirements for epithelial cultures used in toxicity testing are that they retain critical metabolic functions, that all cells be freely accessible to the tested chemicals, and that cells be readily available for replicate sampling to assay chemically-induced effects. Alterations in cellular behavior, such as growth impairment (growth rate and colony-forming efficiency), mutagenesis, neoplastic transformation, and cell

*Department of Pathology, University of North Carolina, School of Medicine, Chapel Hill, North Carolina 27514.

death, may provide sensitive measures of cytotoxicity *in vitro*. In fortunate circumstances, modification of a subcellular macromolecule, enzyme reaction, or organelle may be used as a sensitive and specific indication of toxicity. When alterations of such subcellular events are used as endpoints, it is essential that the modified reaction measured be directly involved in causing or preventing chemical toxicity. In other words, for optimal specificity, the subcellular event measured should be a part of the cellular mechanism that produces altered cellular behavior. These general considerations are illustrated by discussing the advantages and use of liver cultures to evaluate the carcinogenic potential of chemicals.

Theoretical Advantages of Liver Cultures to Screen Carcinogens

Identification of chemicals that are potential etiologic agents of cancer in man is obviously of great importance, since 80% or more of human cancers may be caused by environmental chemicals (*5, 6*). Classic means to identify cancer-causing agents include epidemiologic studies on human populations and whole-life studies in animals. Epidemiologic identification of environmental carcinogens is inefficient and imprecise, being virtually limited to the identification of those compounds to which a small, clearly definable population is exposed or to those compounds that produce rare or unique cancers. Whole-animal studies are also slow and costly, since large groups of animals must be exposed to the tested chemical at different concentrations and routes and observed for the appearance of malignant neoplasms for the remainder of their lives; elaborate control groups are required to evaluate nonspecific toxic side effects of chemicals and to distinguish the occurrence of carcinogen-stimulated and spontaneous cancers. Because they are time-consuming and expensive, whole-animal studies are limited to a few carefully chosen compounds. Compounds should be selected for whole-animal tests by prior short-term screening tests, and if chemicals are to be evaluated comprehensively for carcinogenic potential, screening procedures must be rapid, cheap, and sensitive.

Many relatively simple *in vitro* procedures have been suggested recently for use as carcinogen screens. Most screening procedures are based on the hypothesis that carcinogens are mutagens and act because they modify DNA and lead to mutations, including neoplastic transformation. Most *in vitro* screening procedures, thus, detect primary mutagenic damage to DNA, "repair" of damaged DNA, or the consequences for the cell of imperfect or erroneous repair. *In vitro* screening systems based on this theory have included the induction of mutations in tester strains of bacteria and fungi (*7, 8*) and the induction in mammalian cells (usually fibroblasts) of DNA damage and repair (*9*), sister chromatid exchanges (*10*), mutations (*11*), or neoplastic transformation (*12*). Most potentially carcinogenic chemicals exist outside the body as inactive procarcinogens that require cellular metabolism to form ultimate electrophiles. Since most of the *in vitro* screening systems listed above lack the ability to activate almost all procarcinogens (bacteria and fungi) or have greatly limited ability to activate chemicals of different classes (mammalian fibroblasts), an activating system (the S-9 fraction of homogenized liver containing hepatic microsomes) must be added to them in order to activate and, thus, detect various procarcinogens. Ideally, the activator system and the indicator system should be incorporated in the same cell.

Cultured hepatocytes may provide both activating and indicating capability in the same mammalian cell. Of all tissues, liver has the broadest ability to metabolize chemicals of different classes, possibly being able to metabolically activate all known procarcinogens (*13*). Techniques are available that allow the maintenance in culture of hepatic epithelial cells, which retain many of the functional capabilities of hepatocytes *in vivo*. Preliminary studies suggest that cultured hepatic cells are able to respond to a broad variety of procarcinogens with increased rates of DNA damage and repair, mutagenesis, and neoplastic transformation, and that these subcellular reactions may allow sensitive detection of carcinogenic potential. Much work remains, however, before cultured liver cells can be used routinely to evaluate the carcinogenicity of chemicals. In the remainder of this paper we describe the various types of liver culture, and discuss their advantages and disadvantages for use in *in vitro* systems to screen toxic chemicals.

Characteristics of Hepatic Tissue and Cell Cultures

Epithelial cells from liver can be cultured by the techniques of organ and explant culture, in which some semblance of tissue organization is maintained, or by techniques of primary or continuous (propagable) cell culture, in which dispersed epithelial cells are maintained for varying periods. These types of hepatic cultures differ predominantly in their maintenance of tissue structure, in their retention of hepatic functions, and in their ability to generate new cells by proliferation. These features, as well as the access of cultured cells to

added metabolites, the ease of replicate sampling, and the extent and reproducibility of spontaneous degenerative changes occurring *in vitro*, predicate their relative utility for evaluating chemical toxicity and carcinogenicity.

Cultures of Organized Tissue

Organ Cultures. Organ cultures are made of small pieces of whole liver tissue immersed in or floated on culture fluid and maintained in an atmosphere containing a high concentration of oxygen, typically a mixture of 95% O_2 and 5% CO_2 (*14–17*). Organ cultures are usually placed on a surface composed of a mesh of stainless steel, textile fabric, paper, or coagulated fibrin, which effectively prevents cellular outgrowth. Organoid structure is retained in hepatic organ cultures, and many functions are preserved, both for comparatively short periods. For example, many hepatocytes in organ culture are ultrastructurally virtually normal, and physiological functions, such as glucose metabolism and glycogen synthesis, storage, and release are preserved with appropriate hormonal supplementation (*18–20*). Capability of organ cultures to metabolize chemicals apparently has not been reported. The spontaneous occurrence of cellular degeneration is a major problem to the use of organ culture for evaluating cytotoxicity (*14, 20*). Cellular degeneration that occurs spontaneously appears to result from limited diffusion of oxygen and nutrients into the tissue; necrosis can be limited to some extent by decreasing the size of tissue blocks and by manipulating the levels of insulin and hydrocortisone (*20*). Because of this spontaneous necrosis, the life span of organ cultures of liver is less than one week. A further problem with organ cultures is that their structure is as complex as is the liver *in vivo* (*20*), so that it is impossible to sample just hepatocytes, for example. Because of these disadvantages, organ cultures of liver have not been used widely to study hepatic selective toxicity or to screen chemicals for toxicity.

Explant Cultures. Explant cultures possess some features of both organ and continuous cell cultures. They resemble organ cultures in their origination from a small block of whole liver tissue, in their maintenance in an atmosphere rich in oxygen, and in their retention of some organoid structural features. They differ from organ cultures by virtue of the fact that the tissue block is grown on a surface of glass or plastic that facilitates the outgrowth of cells from the explanted block of tissue (*21–23*). Sheets of cells of various types, including hepatocytes, migrate out of the explanted tissue fragments to form a cytologically complex outgrowth zone (*23*). Formation of thin sheets of cells in outgrowth zones allows cells in these cultures to be continually viewed in the living state by phase microscopy (*22, 23*). Some cellular aggregates in the outgrowth zone retain an organoid structure, even though their outward migration from the explant is attended by a self-limited burst of cell proliferation (*24, 25*). For example, bile canaliculi and ducts are preserved in a morphologically normal configuration. Some hepatic functions are retained, such as the synthesis of selected serum proteins (Grisham, J. W., unpublished observations), the hormonally predicated synthesis and release of glycogen (*20*), the metabolism of benzo(a)pyrene (*26*), and the uptake, metabolism, and secretion into bile canaliculi of chemicals that are metabolized in a manner similar to bilirubin (Grisham, J. W., unpublished observations). Under optimal circumstances, cells in outgrowth zones of explant cultures may remain viable for several weeks (*23*).

The major disadvantages of explant cultures pertain to their small size and to the difficulty of manipulating the cells that they contain. For biochemical analyses, microdissection must be used to select the desired type of cells from the complex mixture in the outgrowth zones, and sensitive micromethods must be employed to measure metabolic reactions on the small number of cells available. The major advantages of explant cultures are the retention of organoid structure and, perhaps, function, combined with reproducible cell proliferation. Their growth configuration as thin cellular layers facilitates the continuous microscopic examination of living cells.

Cultures of Dispersed Cells

Cultures of dispersed hepatic epithelial cells, either primary or propagated, recently have been established in many laboratories from livers of several species. Typically hepatic cells are enzymatically dispersed either by perfusing livers *in situ* with solutions containing collagenase alone, or combined with hyaluronidase, or by treating minced liver with similar enzymic solutions *in vitro* (*27, 28*). Hepatocytes for primary cultures are concentrated and partially separated from other cells by gravity or low-speed centrifugal sedimentation. Continuous lines of epithelial cells are established by culturing cell mixtures until discrete colonies form, which are then subcultured. Propagable cell strains are started by cloning from single cells, either in primary culture or in secondary culture after establishment of mass primaries. Hepatic epithelial lines have also been established by isolating and subculturing cells from the outgrowth zone of explant cultures.

Hepatocyte Primary Cultures. Enzymatically isolated hepatocytes can be maintained in primary culture by three major methods, monolayer culture (*29*), suspension culture (*30*), or culture on floating collagen membranes (*31*); each of these methods has advantages and limitations. In the most widely used technique, hepatocytes are plated directly onto plastic or glass dishes (with or without a coating of soluble collagen) (*29, 31–34*). After 24 hr, viable cells attach to the substrate and flatten, and by the second culture day attached cells form a nearly continuous monolayer. Populations of hepatocytes in monolayer culture decrease continuously as a result of spontaneous cell death (*35*), and for optimal use, cells should be used for study during the first three or four days *in vitro*. Monolayer cultures are useful for morphologic study of living cells and for other techniques that require thinly spread cells (such as autoradiography). Sampling of cells of monolayer cultures for biochemical analysis is easily accomplished by dispersing attached cells with a rubber spatula. Suspension cultures, in which cells are maintained in a suspended state by continual mixing, can be established in truly mass quantities, each containing billions of cells, which maintain a high level of functional integrity for three to five days (*30*). Cells in suspension culture aggregate to form spherical masses, requiring that histologic sections be made to apply morphological techniques (*36*), such as autoradiography. Replicate sampling is readily accomplished by pipetting aliquots of suspended cells. Collagen membrane cultures are formed by plating isolated hepatocytes on a freshly made collagen gel. During the first few days the collagen gel shrinks to about one-sixth of its original diameter, enfolding the hepatocytes and floating on the surface of the culture medium. Hepatocytes floating on the collagen membrane remain functionally viable for up to 20 days (*37*). Collagen membrane cultures are small and somewhat difficult to sample for biochemical and morphologic studies.

The surface and internal structure of carefully isolated hepatocytes is virtually normal, and this is maintained in viable cells in primary culture (*36–38*). Abutting cells reform attachment complexes and bile canaliculi in culture. In degenerating cells, permeability to trypan blue is correlated with loss of surface microvilli, fragmentation of the endoplasmic membrane, and swelling of mitochondria.

In separate studies, hepatocytes in primary culture have been shown to synthesize albumin, hemopexin, fibrinogen, ceruloplasmin, α-1-antitrypsin, α-fetoprotein, transferrin, the third component of complement, heme, fatty acids, and cholesterol (*39, 40*). Hepatocytes in primary suspension cultures synthesize albumin at the *in vivo* rate (*30*), and fatty acid synthesis is under feedback control (*40*). Cultured hepatocytes can also synthesize glucose from lactate, and they synthesize, store and release glycogen on appropriate hormonal stimulation (*40*). Cultured hepatocytes conjugate bile acids and metabolize several drugs (*40*).

A major defect of hepatocytes in most types of primary culture is the deficiency of cytochrome P-450 (*41*). Cytochrome P-450 rapidly breaks down in hepatocytes in monolayer culture, decreasing to 10–20% of the level *in vivo* by 24 hr (*32*). Although the mechanism responsible for the breakdown of cytochrome P-450 in freshly isolated hepatocytes is not clear, it may result from autocatalytic lipid peroxidation of microsomal membranes. Breakdown of cytochrome P-450 is associated with increased levels of heme oxygenase (*39, 40*). Aryl hydrocarbon hydroxylase activity is markedly reduced, and metabolism of drugs is variably decreased (*41*). Hepatocytes cultured either in suspension or on floating collagen membranes maintain higher cellular levels of cytochrome P-450 than do cells in monolayer cultures (*42*); in suspension cultures, aryl hydrocarbon hydroxylase levels may be maintained at *in vivo* levels (*40*). Treatment of adult hepatocytes on floating collagen membranes with either phenobarbital (10^{-4}–$10^{-3}M$) or 3-methylcholanthrene (2–5μM), induces up to 2-fold increases in cytochrome P-450 and P-448. It may be possible to prevent catabolism of cytochrome P-450 in hepatocytes in primary culture by supplementing medium with hormones and cofactors (*43*).

Comprehensive, direct examination of the capability of hepatocytes in primary culture to metabolize chemicals is sparse. Fetal hepatocytes in monolayer culture slowly convert benzo(a)pyrene to more polar metabolites (*44*) and adult hepatocytes in primary culture convert this chemical to a variety of primary and secondary metabolites (*45*). Adult hepatocytes in proliferating primary cultures convert *N*-2-acetylaminofluorene to *N*-hydroxyacetylaminofluorene (*46*). Degradation of cytochrome P-450 in conventional monolayer cultures suggests that the metabolism of at least some chemicals will be impaired and this has been coroborated in a study in which metabolism of several drugs was found to be variably affected (*41*). High levels of cytochrome P-450 in hepatocytes in suspension cultures (*40*) and hormone-supplemented monolayers (*43*) indicate that these cells may be able to actively metabolize chemicals; this is supported by the observation that they metabolize diphenylhydantoin (*47*) and aflatoxin B_1 (*43*) at rates comparable to the liver *in vivo*. Indirect evidence that hepatocytes in monolayer culture can

activate several procarcinogens is suggested by studies in which these chemicals elicited unschedules DNA synthesis in exposed cells (*48*).

Adult hepatocytes in conventional monolayer primary culture do not proliferate (*49*), but they may undergo at least one proliferative cycle when grown under modified conditions (*34*). In conventional monolayer cultures, cells isolated during the G_1 phase *in vivo* do not enter S phase *in vitro*, although cells isolated while replicating their DNA can complete this process *in vitro* (*49*). The question of cell proliferation in suspension cultures apparently has not been studied.

Because of their somewhat unstable functional status and brief life span *in vitro*, coupled with their limited ability to proliferate in culture, the direct determination of cytotoxicity in primary cultures of hepatocytes is not easy. Analysis of cloning efficiency is not possible, since hepatocytes in primary cultures are not clonogenic. Evaluation of cytotoxicity by determining the decrease in number of viable cells, the release of cellular enzymes, the release of ^{51}Cr, or trypan blue uptake is complicated by the fact that appreciable numbers of hepatocytes die under basal conditions in primary cultures. Furthermore, since each hepatocyte primary culture must be established with cells freshly isolated from liver, these cultures may show considerable functional differences from batch to batch that reflect differences in the conditions of animals and variations in the isolation technique.

Continuous Lines and Strains of Hepatic Epithelial Cells. Several lines (not clonally derived) and strains (cloned from single cells) of propagable epithelial cells from liver have been developed (*50–62*). Different continuously culturable hepatic epithelial cell lines and strains vary in some respects, but they possess general similarities. They are composed of structurally simple cells that grow as coherent epithelial sheets joined by attachment complexes (*50, 57, 62*). They remain diploid if carefully subcultured (*63*), but malignant transformation has occurred spontaneously and after treatment with chemicals and viruses (see below). Cytoplasmic organelles differ considerably from similar structures in hepatocytes (*57, 62, 64*). Endoplasmic reticulum is sparse, mitochondria are small, and peroxisomes are few or are lacking entirely. Lysosomes are typically numerous, and golgi apparatuses are prominent. In keeping with the presence of gap junctions, adjacent cells in sheets are electronically coupled (*51*). Electronic coupling is lost in malignantly transformed cells (*51*).

On subculture, epithelial cells attach readily to substrates with an attachment efficiency of over 85%; however, the colony forming ability is low, varying from less than 1% to about 15% (*50, 58, 60, 65*). Colony-forming efficiency and cell proliferation are markedly concentration-dependent; single cells form clones only when plated at relatively high concentrations, but growth is poor at low plating densities (*65*). At optimal densities, doubling times of different lines and strains varies from 24 to 60 hr (*49, 57, 59, 61*). Cell proliferation is suppressed at high population densities, which in diploid lines occurs at from 2 to 20 $\times$ 10^4 cells/cm^2 (*58, 65*). The saturation density is typically raised by 2- to 5-fold in transformed sublines (*57*; Grisham, J. W., unpublished observations).

Continuous lines and strains of hepatic epithelial cells retain a few, but not a large combination of hepatocyte-like functional properties. Cells from various lines or strains synthesize and secrete one or more serum proteins, including albumin, transferrin, α-fetoprotein, and some clotting factors (*55, 58, 64*). Tyrosine aminotransferase is present, but it is not inducible with steroids in most, if not all, diploid lines. Several aneuploid lines, however, have inducible tyrosine aminotransferase (*52*). Similarly glycogen synthesis and storage on hormonal stimulation appears to be a property of only some aneuploid lines (*65*). Levels and forms of aldolase isoenzymes are uniformally fetal in type (*66*).

The ability to metabolize exogenous chemicals varies markedly between lines, and mixed function oxidation is practically limited to those reactions involving cytochrome P-448 (*67–69*). In a few lines, aryl hydrocarbon hydroxylase is present at levels that approach those in intact liver (*69*); however, this is unusual and typically the constitutive level of this enzyme is much lower (*67–69*). With repeated subculture the constitutive level of aryl hydrocarbon hydroxylase may fall (*69*). Metabolic activity toward benzo(a)pyrene may be induced greatly by prior exposure to methylcholanthrene, but not to phenobarbital, (*68, 69*).

The *in vivo* origin of propagable hepatic epithelial cells is uncertain. Some workers have proposed that these cells represent functionally and structurally simplified (dedifferentiated) hepatocytes. However, studies in our laboratory indicate that they are derived from small clonogenic cells, morphologically distinct from hepatocytes *in vivo* (*62*). This evidence suggests that these cells possess some properties of stem cells in other tissues, and that they may be derived from terminal bile ductules.

Genotoxicity of Chemicals for Hepatocytes in Primary Culture

Detection of unscheduled incorporation of radiolabeled thymidine into DNA as a measure of

DNA repair, either by autoradiography (*48, 70*) or liquid scintillation counting (*71, 72*), has been used to assay the genotoxic action of chemicals on cultured primary hepatocytes *in vitro*. At concentrations greater than $10^{-5}M$, the potent carcinogen aflatoxin B_1 produces more than four times the nuclear grains in autoradiographs prepared from hepatocytes in monolayer cultures than do noncarcinogenic chemicals (*48, 70*). At concentrations of aflatoxin B_1 ranging from 10^{-7} to $10^{-3}M$, the magnitude of DNA repair was roughly dose-dependent, although there was considerable variation between consecutive assays using hepatocytes isolated from different animals at different times (*48, 70*). The strong carcinogens *N*-2-acetylaminofluorene and 7, 12-dimethylbenz(a)anthracene, at concentrations of $10^{-3}M$, also produced unscheduled DNA synthesis at more than four times the basal level (*48, 70*). The potent carcinogens *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 3-methyl-4-dimethyl-aminoazobenzene, and dimethylnitrosamine initially failed to produce more nuclear grains than did the noncarcinogens *N*-4-acetylaminofluorene, 4-aminobenzene, and dimethylformamide (*70*). Furthermore, the weak carcinogens aflatoxin B_2 and benz(a)anthracene also failed to elicit more unscheduled DNA synthesis than did the noncarcinogenic chemicals (*70*). In subsequent studies in which the conditions of chemical exposure and assay of unscheduled DNA synthesis were modified, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, dimethylnitrosamine, and aflatoxin B_2 gave positive results, while benz(a)anthracene remained negative (*48*).

Unscheduled DNA synthesis also has been detected by liquid scintillation counting of DNA isolated from hepatocytes exposed to *N*-2-acetylaminofluorene in primary hepatocyte cultures on floating collagen membranes, in suspension, or in monolayers (*71, 72*). Exposure of hepatocytes on floating collagen membranes or in monolayers to $2 \times 10^{-4}M$ *N*-2-acetylaminofluorene produced a 2.5-fold increase in the specific activity of DNA as compared to controls (*71*). Exposure to *N*-2-acetylaminofluorene at $2 \times 10^{-5}M$ resulted in a 1.2- to 1.6-fold increase in DNA specific activity, suggesting that the effect was concentration-dependent (*72*). Benzo(a)pyrene, aflatoxin B_1, dimethylnitrosamine, 3'-methyldimethylaminoazobenzene, and saffrole at 10^{-5}–$10^{-8}M$ increased unscheduled incorporation of radioactive thymidine into DNA by 1.5- to 4-fold over the control values (*71*). At $10^{-3}M$ and $10^{-4}M$, these compounds were so toxic that they killed exposed hepatocytes outright, preventing evaluation of DNA repair (*71*).

Although there is a fairly good correlation between the production of unscheduled DNA synthesis (repair) in hepatocytes in primary culture and the known relative carcinogenicity of chemicals for hepatocytes *in vivo*, results have varied considerably and methods have had to be modified to place some known carcinogens into the positive category. This *in vitro* variation may result from the marked degradation of cytochrome P-450 and the consequent impairment of mixed function oxidation, which effects many isolates of primary hepatocytes, or it may result from the fact that extent of repair synthesis of DNA in the small excised patches created by some chemicals, such as *N*-methyl *N'*-nitro-*N*-nitrosoguanidine, may be near the level of resolution of the methods used. The sensitivity of methods employing both scintillation counting and autoradiography are dependent on the stimulation of sufficient repair synthesis in DNA of affected cells to be readily quantifiable. Assays based on evaluation of DNA repair synthesis are apt to detect most efficiently those chemicals that produce large gaps; however, the sizes of gaps produced in DNA by different chemicals varies over a wide range, some chemicals producing tiny gaps whose filling causes only modest increases in thymidine incorporation into DNA (*73*). In addition, these methods probably require a large number of damaged sites to give labeling detectable above background. Evaluation of DNA repair synthesis seems to detect most efficiently the effects of those chemicals that produce large gaps over much of the genome.

Direct binding of chemical metabolites to DNA, removal of altered bases from DNA, or the creation and sealing of breaks in DNA strands apparently has not been examined after exposure of hepatocytes in primary culture to various chemicals. Analysis of strand discontinuity should provide a more sensitive method to detect DNA damage and repair than quantitation by DNA repair synthesis, since the magnitude of this effect is not influenced by the size of the gap created. Even a single-strand break without base removal would lower the molecular weight of DNA as much as would a gap of 1000 nucleotides, although the former would elicit no repair synthesis.

Nitroso compounds and polycyclic hydrocarbons are directly genotoxic for cultured hepatic epithelial cells in continuous culture (*74*). *N*-Methyl-*N*-nitrosourea caused dose-dependent DNA strand breaks that were quickly repaired, and the procarcinogens dimethylnitrosamine, *N*-2-acetylaminofluorene, aflatoxin B_1, and dimethylbenzanthracene led to DNA strand breaks in hepatic epithelial cells exposed to these chemicals in the presence of $0.4mM$ chloroquine (*74*). Chloroquine is presumed to potentiate observation of DNA breakage by pre-

venting repair and causing breaks to accumulate with time of exposure.

Cultured hepatic epithelial cells mutate to develop resistance to 8-azaguanine when exposed to methyl methanesulfonate, aflatoxin B_1, dimethylbenzanthracene, dimethylnitrosamine, and *N*-2-acetylaminofluorene (*75*). The mutation rate in response to methyl methanesulfonate is enhanced when cells are exposed during S phase (330 azaguanine resistant colonies per 10^6 proliferating cells compared to 32 resistant colonies per 10^6 nonproliferating cells) (*75*).

Neoplastic Transformation of Hepatic Epithelial Cells by Chemicals *in Vitro*

Morphological transformation of hepatocytes in hepatic organ cultures exposed to *N*-2-acetylaminofluorene has been described briefly (*76*), but the results have not been fully detailed nor have they been corroborated.

Malignant transformation of cultured hepatic epithelial cells has been accomplished by several investigators by exposing cells *in vitro* to several chemical carcinogens (*77–83*), as well as to SV-40 virus (*58*). These studies demonstrated that transformed hepatic epithelial cells, when strains or "pure" lines are used, produce adenocarcinomas on back-transplantation into isogeneic animals and not sarcomas, as is the situation with transformed fibroblasts. Chemical transformants from some nonclonally derived hepatic epithelial cell lines have produced sarcomas or carcinosarcomas on back transplantation, reflecting the fact that these cultures included a mixed population of epithelial and mesenchymal cells (*79*). Carcinogens successfully employed to transform hepatic epithelial cells *in vitro* are dimethylnitrosamine (*78, 79, 82*), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (*79, 82*), *N*-methyl-*N*-nitrosourea (*78, 79, 82*), *N*-hydroxyacetylaminofluorene (*78*), *N*-acetoxyacetylaminofluorene (*81*), 4-nitroquinoline-*N*-oxide (*77*), aflatoxin B_1 (*78, 80*), 3-methylcholanthrene (*81*), 3-methyldimethylaminoazobenzene (*81*), 7,12-dimethylbenzanthracene (*78*), benzo(a)pyrene (*83*), and methylazoxymethanol (*83*). Thus, most classes of carcinogens, including both active and procarcinogens, are able to transform hepatic epithelial cells under the conditions studied. A major handicap to the facile study of neoplastic transformation in hepatic epithelial cells stems from the fact that the morphology of transformed cells does not differ greatly from that of the untransformed diploid stock, unlike the situation with fibroblasts. This condition requires that the evaluation of transformation *in vitro* be based on altered behavior, such as ability to grow in soft agar or to produce tumors *in vivo*, and it prevents the clonal analysis of transformation.

Reported studies on transformation of cultured hepatic epithelial cells all have been performed by repeated or continuous exposure of cells in mass cultures to chemicals, followed by a period of subculture in the absence of the chemical agent, and blind back-transplantation (without being able to identify transformants *in vitro*) of aliquots into test hosts. This experimental procedure makes it impossible to prove that transformation resulted from induction rather than selection. The dilemma is further enhanced by the occurrence of spontaneous malignant transformation in cultured hepatic epithelial cells (*58, 63, 69, 77, 78, 82–87*). Growth of epithelial cells under crowded (confluent) conditions without regular refeeding with fresh medium, causes them to transform (*63*). The risk of spontaneous transformation appears to increase after 10 to 15 subculture generations (*87*), but spontaneous transformation may occur during the first passage *in vitro* (Charlton, R. K., unpublished observations). These observations emphasize the importance of using continuously propagated hepatic epithelial cells at early culture generations and of growing them under conditions that ensure adequate nutrition. Aliquots of low generation cells can be stored indefinitely in liquid nitrogen; plating efficiency of thawed cells is over 85%, and the cloning efficiency approximates that of cells that have not been frozen.

The "kinetics" of transformation in hepatic epithelial cells seem to differ from that in cultured fibroblasts exposed to carcinogens *in vitro*. More time appears to be necessary for transformants to be expressed in epithelial cells than in fibroblasts (*78, 79*). It is not clear whether initiation or augmentation of the initiated population, or both processes, are slower in epithelial cells. Although the meaning of this difference is unknown presently, it possibly reflects important biologic differences in the transformation process between epithelial and mesenchymal cells. Further examination of the process of neoplastic transformation in hepatic epithelial cells is warranted to provide better understanding of this important pathobiologic process.

Although hepatic epithelial cell lines and strains transform in response to *in vitro* exposure to a variety of carcinogens, the slowness of epithelial transformation limits the application of this potentially useful system as a rapid chemical screen.

Other Measurements of Cytotoxicity in Hepatic Tissue and Cell Cultures

Organ cultures of liver have been used to assay the potential and mechanisms of hepatotoxicity of several chemicals; morphological and biochemical measurements were used to evaluate the effect of aflatoxin B_1 (*90, 91*), tetracycline (*92*), pyrrolizidine alkaloids (*93–95*), and sulfonylureas (*96*).

Hepatocytes in primary cultures have not been widely used to attempt to assay toxicity of noncarcinogenic chemicals. Hepatotoxicity of selected metallic salts has been detected by evaluating permeability to trypan blue (*88*). Some hepatotoxic drugs are said to cause increased rates of enzyme-release by cultured hepatocytes (*89*).

As compared to cultured fibroblasts, hepatic epithelial cells in continuous culture demonstrate a differential toxic response to many agents, apparently because of differing metabolic capabilities. Presumably because it acts directly without requiring metabolic activation, α-naphthylisothiocyanate kills HeLa and 3T6 cells with the same efficiency as it does hepatic epithelial cells (*96*). In contrast, benzo(a)pyrene is not toxic for A9 or HeLa cells, but it does kill hepatic epithelial cells (*68, 98*); this differential response probably occurs because of the inability of the former cells to convert this chemical to toxic metabolites, whereas the hepatic epithelial cells could do so. In support of this opinion, 7,8-benzoflavone and certain estrogenic and androgenic steroids significantly reduce the killing of hepatic epithelial cells by 7,12-dimethylbenzanthracene (*99*). Aflatoxin B_1 killed 84% of a continuous fibroblast line but only 19% of a line of hepatic epithelial cells when used at a concentration of 50 μg/ml for 48 hr (*100*), presumably because the epithelial cells can more effectively catabolize this chemical.

Conclusion

The present state-of-the-art in the field of hepatic tissue and cell culture limits the immediate application of these techniques to the evaluation of chemical toxicity. Two major problems, for example, impede the use of hepatocytes in primary culture to screen chemicals for carcinogenic potential. The first is the functional variability between batches of cells isolated at different times; the second is the general deterioration of mixed function oxidation in these cells. Studies are needed to define methods to isolate cells with reproducible functions and to measure directly the ability of isolated hepatocytes to metabolize chemicals of different classes. Much work remains to validate the use of any of the hepatic culture methods for *in vitro* toxicity testing; validation will require the direct comparison of results of studies in culture systems *in vitro* and in intact livers *in vivo*. Before hepatic cultures can be used to screen chemicals for toxicity, their sensitivity, precision, and reproducibility must be thoroughly and critically tested.

Propagable cultures (lines and strains) of hepatic epithelial cells have, for the first time, allowed the process of carcinogenesis (per se), rather than sarcomagenesis to be studied *in vitro*. This is a signal accomplishment and may lead ultimately to greater insights into the causes and mechanisms of cancer development. Further study of this important pathobiologic process is justified, even though hepatic epithelial cells in continuous culture appear to have only limited value for rapid screening of chemicals.

Organoid cultures do not appear to be readily adaptable to the evaluation of hepatotoxicity *in vitro*. However, this application of organ and explant cultures of liver has not been explored widely in recent years, at a time when organotypic cultures, generally, are receiving much attention in cancer research.

Despite these problems, the future appears bright. Each session in the laboratory and each trip to the library brings forth new insights concerning the isolation and *in vitro* maintenance of hepatocytes and other hepatic cells that retain a higher degree and broader variety of functional abilities. The field is rapidly advancing, and it does not appear unreasonable to predict the future wide use of hepatic tissue and cell cultures to evaluate the toxicity of chemicals and the cellular mechanisms of their toxicity.

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REFERENCES

1. Health Hazards in the Human Environment. World Health Organization, Geneva, 1972, p. 168.
2. Dawson, M., and Dryden, W. F. Tissue culture in the study of the effects of drugs. J. Pharm. Sci. 56: 545 (1967).
3. Schindler, R. Use of cell culture in pharmacology. Ann. Rev. Pharm. 9: 393 (1969).
4. Harris, C. C., et al. Localization of benzo(a)pyrene-^{3}H and alterations in nuclear chromatin caused by benzo(a)pyrene-ferric oxide in the hamster respiratory epithelium. Cancer Res. 33: 2842 (1973.
5. Higginson, J. Present trends in cancer epidemiology. Can. Cancer Conf. 8: 40 (1969).
6. Wynder, E. L., and Gori, G. B. Contribution of the environment to cancer incidence: an epidemiologic exercise. J. Nat. Cancer Inst. 58: 825 (1977).

7. McCann, J., et al. Detection of carcinogens as mutagens: Bacterial tester strains with R factor plasmids. Proc. Nat. Acad. Sci. (U. S.) 72: 979 (1975).
8. Brusick, D., and Andrews, H. Comparison of the genetic activity of dimethylnitrosamine, ethyl methane sulfonate, 2-acetylaminofluorene, and ICR-170 in *Saccharomyces cerevisiae* strains D_3, D_4, and D_5 using *in vitro* assays with and without metabolic activation. Mutat. Res. 26: 491 (1975).
9. Stich, H. F., et al. The use of DNA repair in the identification of carcinogens, procarcinogens, and target tissue. Can. Cancer Conf. 10: 83 (1974).
10. Perry, P., and Evans, H. J. Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature 258: 121 (1975).
11. Arlett, C. F., et al. A comparison of the 8-azaguanine and ouabain-resistance systems for the selection of induced mutant Chinese hamster cells. Mutat. Res. 33: 261 (1975).
12. DiPaolo, J. A., Donovan, P. J., and Nelson, R. L. Quantitative studies on *in vitro* transformation by chemical carcinogens. J. Nat. Cancer Inst. 42: 867 (1969).
13. Weisburger, J. H., and Williams, G. M. Metabolism of chemical carcinogens. In: Cancer, A Comprehensive Treatise, Vol. 1, F. F. Becker, Ed., Plenum Press, New York, 1975, p. 185.
14. Laufs, R., and Walker, W. Metabolic and virologic studies in primate liver organ cultures. Proc. Soc. Exp. Biol. Med. 133: 1006 (1970).
15. Campbell, A. K., and Hales, C. N. Maintenance of viable cells in an organ culture of mature rat liver. Exptl. Cell Res. 68: 33 (1971).
16. Kalus, M., et al. Marmoset liver organ culture infected with herpes virus. Exptl. Mol. Pathol. 8: 388 (1968).
17. Parsa, I. Liver differentiation in a chemically defined medium. Morphologic and enzymatic comparisons of *in utero* rat liver and liver rudiment growing in organ culture. Am. J. Pathol. 76: 107 (1974).
18. Monder, C., and Coufalik, A. Influence of cortisol on glycogen synthesis and gluconeogenesis in fetal rat liver in organ culture. J. Biol. Chem. 247: 3608 (1972).
19. Eisen, H. J., Glinsmann, W. J., and Sherline, P. Effect of insulin on glycogen synthesis in fetal rat liver in organ culture. Endocrinol. 92: 584 (1973).
20. Reed, G. B., and Grisham, J. W. Insulin and hydrocortisone effects on viability and glycogen stores of postnatal rat liver culture. Lab. Invest. 33: 298 (1975).
21. Sandström, B. A method of cultivating morphologically highly differentiated liver epithelium *in vitro*. Acta Soc. Med. Upsal. 69: 209 (1964).
22. Rose, G. G., Kumegawa, M., and Cattoni, M. The circumfusion system for multipurpose culture chambers, II. The protracted maintenance of differentiation of fetal and newborn mouse liver in vitro. J. Cell Biol. 39: 430 (1968).
23. Alexander R. W., and Grisham, J. W. Explant culture of rat liver. I. Method, morphology and cytogenesis. Lab. Invest. 22: 50 (1970).
24. Grisham, J. W. Kinetics of cell proliferation in explant cultures of rat liver. J. Cell Biol. 47: 79a (1970).
25. Rabes, H., and Jantsch, B. Proliferationskinetik der Hepatocyten in primären Leberkulturen. Z. Zellforsch. 112: 414 (1971).
26. Cutroneo, K. R., and Bresnick, E. Induction of benzypyrene hydroxylase in fetal liver explants by flavones and phenobarbital. Biochem. Pharm. 22: 675 (1973).
27. Berry, M. N., and Friend, D. S. High-yield preparations of isolated rat liver parenchymal cells: a biochemical and fine structural study. J. Cell Biol. 43: 506 (1969).
28. Seglen, P. O. Preparation of rat liver cells. III. Enzymatic requirements for tissue dispersion. Exptl. Cell Res. 82: 391 (1973).
29. Williams, G. M. Primary and long-term culture of adult rat liver epithelial cells. Meth. Cell Biol. 14: 357 (1976).
30. Jeejeebhoy, K. N., et al. Albumin, fibrinogen, and transferrin synthesis is isolated rat hepatocyte suspensions. Biochem. J. 146: 141 (1975).
31. Michalopoulos, G., and Pitot, H. C. Primary culture of parenchymal liver cells on collagen membranes. Morphological and biochemical observations. Exptl. Cell Res. 94: 70 (1975).
32. Bissell, D. M., Hammaker, L. E., and Meyer, V. A. Parenchymal cells from adult rat liver in nonproliferating monolayer culture. I. Functional studies. J. Cell Biol. 59: 722 (1973).
33. Bonney, R. J. Adult liver parenchymal cells in primary culture: characteristics and cell recognition standards. *In Vitro* 10: 130 (1974).
34. Leffert, H. L., and Paul, D. Studies on primary cultures of differentiated fetal liver cells. J. Cell Biol. 52: 559 (1972).
35. Laishes, B. A., and Williams, G. M. Conditions affecting primary cell cultures of functional adult rat hepatocytes. I. The effects of insulin. *In Vitro* 12: 521 (1976).
36. Phillips, M. J., et al. Ultrastructural and functional studies of cultured hepatocytes. Lab. Invest. 31: 533 (1974).
37. Michalopoulos, G., et al. Cytochrome P-450 induction by phenobarbital and 3-methylcholanthrene in primary cultures of hepatocytes. Science 193: 907 (1976).
38. Chapman, G. S., et al. Parenchymal cells from adult rat liver in nonproliferating monolayer culture. II. Ultrastructural studies. J. Cell Biol. 59: 735 (1973).
39. Bissell, D. M. Study of hepatocyte function in cell culture. Progr. Liver Dis. 5: 59 (1976).
40. Jeejeebhoy, K. N., and Phillips, M. J. Isolated mammalian hepatocytes in culture. Gastroenterology 71: 1086 (1976).
41. Guzelian, P. S., and Bissell, D. M. Effect of cobalt on synthesis of heme and cytochrome P-450 in the liver. Studies of adult rat hepatocytes in primary monolayer culture *in vivo*. J. Biol. Chem. 251: 4421 (1976).
42. Michalopoulos, G., et al. Interactions of chemical carcinogens and drug metabolizing enzymes in primary cultures of hepatic cells from the rat. Am. J. Pathol. 85: 755 (1976).
43. Decad, G. M., Hsieh, D. P. H., and Bayrd, J. L. Maintenance of cytochrome P-450 and metabolism of aflatoxin B_1 in primary hepatocyte cultures. Biochem. Biophys. Res. Commun. 78: 279 (1977).
44. Nebert, D. W., and Gielen, J. E. Aryl hydrocarbon hydroxylase induction in mammalian liver cell culture. II. Effects of actinomycin D and cyclohexamide on induction process by phenobarbital or polycyclic hydrocarbons. J. Biol. Chem. 246: 5199 (1971).
45. Burke, M. D., Vadi, H., Jernström, B., and Orrenius, S. Metabolism of benzo(a)pyrene with isolated hepatocytes and the formation and degradation of DNA-binding derivatives. J. Biol. Chem. 252: 6424 (1977).
46. Leffert, H. L., Moran, T., and Boorstein, R. Procarcinogen activation and hormonal control of cell proliferation in differentiated adult rat liver cell cultures. Nature 267: 58 (1977).
47. Inaba, T., et al. Isolated rat hepatocytes as a model to study drug metabolism: dose-dependent metabolism of diphenylhydantoin. Life Sci. 16: 1227 (1975).
48. Williams, G. M. Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. Cancer Res. 37: 1845 (1977).
49. Yager, J. D., et al. DNA synthesis in primary cultures of parenchymal cells isolated from regenerating rat liver. In: Liver Regeneration after Experimental Injury, R. Lesch

and W. Reuttner, Eds., Stratton, New York, 1975, p. 148.
50. Coon, H. G. Clonal culture of differentiated cells from mammals: rat liver cell culture. Carnegie Inst. Washington Yearbook 67: 419 (1968).
51. Borek, C., Hagashino, S., and Lowenstein, W. R. Intercellular communication and tissue growth. IV. Conductance of membrane junctions of normal and cancerous cells in culture. J. Memb. Biol. 1: 274 (1969).
52. Gerschenson, L. E., et al. Tyrosine transaminase induction by dexamethasone in a new rat liver cell line. Science 170: 859 (1970).
53. Williams, G. M., Weisburger, E. L., and Weisburger, J. H. Isolation and long-term cell culture of epithelial-like cells from rat liver. Exptl. Cell Res. 69: 106 (1971).
54. Iype, P. T. Cultures from adult rat liver cells. 1. Establishment of monolayer cell-cultures from normal rat liver. J. Cell Physiol. 78: 281 (1971).
55. Kaighn, M. E., and Prince, A. M. Production of albumin and other serum proteins by clonal cultures of normal human liver. Proc. Nat. Acad. Sci. (U. S.) 68: 2396 (1971).
56. Rutzky, L. P., Taylor, W. G., and Pumper, R. W. In vitro stimulation of rat liver cells by homologous partial hepatectomy serum. *In Vitro* 7: 1 (1971).
57. Lambiotte, M., Susor, W., and Cahn, R. D. Morphological and biochemical observations on mammalian liver cells in culture. Isolation of a clonal strain from rat liver. Biochimie 54: 1179 (1972).
58. Diamond, L., et al. The WIRL-3 rat liver cell lines and their transformed derivatives. Cancer Res. 33: 2627 (1973).
59. Chessebeuf, M., et al. Long-term cell culture of rat liver epithelial cells retaining some hepatic functions. Biochimie 56: 1365 (1974).
60. Bausher, J., and Shaeffer, W. I. A diploid rat liver cell culture. 1. Characterization and sensitivity to aflatoxin B_1. *In Vitro* 9: 286 (1974).
61. Masuji, H., Nakabayashi, H., and Sato, J. Long-term cultivation of two diploid epithelial cell lines derived from normal rat liver cells. Acta Med. Okayama 28: 281 (1974).
62. Grisham, J. W., Thal, S. B., and Nägel, A. Cellular derivation of continuously cultured epithelial cells from normal rat liver. In: Gene Expression and Carcinogenesis in Cultured Liver. L. E. Gerschenson and E. B. Thompson, Eds., Academic Press, New York, 1975, p. 1.
63. Borek, C. Neoplastic transformation *in vitro* of a clone of adult liver epithelial cells into differentiated hepatoma-like cells under conditions of nutritional stress. Proc. Nat. Acad. Sci. (U.S.) 69: 956 (1972).
64. Coon, H. G., and Manasek, F. J. Electron microscopy of cultured cells. Carnegie Inst. Washington Yearbook 68: 540 (1969).
65. Gerschenson, L. E., and Casanello, C. Metabolism of rat liver cells cultured in suspension: insulin and glucagon effects on glycogen level. Biochem. Biophys. Res. Commun. 33: 584 (1968).
66. Walker, P. R., et al. Pyruvate kinase, hexokinase, and aldolase isozymes in rat liver cells in culture. *In Vitro* 8: 107 (1972).
67. Whitlock, J. P., and Gelboin, H. V. Arylhydrocarbon (benzo[a]pyrene) hydroxylase induction in rat liver cells in culture. J. Biol. Chem. 249: 2616 (1974).
68. Bartholomew, J. C., et al. Benzo(a)pyrene effects on mouse epithelial cells in culture. Cancer Res. 35: 851 (1975).
69. Idoine, J. B., et al. Rat liver cells in culture: Effects of storage, long-term culture, and transformation on some enzyme levels. *In Vitro* 12: 541 (1976).
70. Williams, G. M. Carcinogen-induced DNA repair in primary rat liver cell cultures: A possible screen for chemical carcinogens. Cancer Letters 1: 231 (1976).
71. Michalopoulos, G., et al. Unscheduled DNA synthesis induced in hepatocellular suspensions and primary cultures of hepatocytes by procarcinogens. Proc. Am. Assoc. Cancer Res. 18: 246 (1977).
72. Yager, J. D., and Miller, J. A. DNA repair in primary cultures of rat hepatocytes. Proc. Am. Assoc. Cancer Res. 18: 159 (1977).
73. Regan, J. C. and Setlow, R. B. Two forms of repair in the DNA of human cells damaged by chemical carcinogens and mutagens. Cancer Res. 34: 3318 (1974).
74. Williams, G. M. The study of chemical carcinogens using rat liver cells. In: Gene Expression and Carcinogenesis in Cultured Liver. L. E. Gerschenson and E. B. Thompason, Eds., Academic Press, New York, 1975, p. 480.
75. Williams, G. M. The use of liver epithelial cultures for the study of chemical carcinogens. Am. J. Pathol. 85: 739 (1976).
76. Parsa, I. Morphological transformation of oran cultured embryonic rat liver by *N*-2-fluorenylacetamide (2-FAA). Fed. Proc. 33: 595 (1974).
77. Katsuta, H., and Takaoka, T. Carcinogenesis in tissue culture. XIV. Malignant transformation of rat liver parenchymal cells treated with 4-nitroquinoline-1-oxide in tissue culture. J. Nat. Cancer Inst. 49: 1563 (1972).
78. Williams, G. M., Elliott, J. M., and Weisburger, J. H. Carcinoma after malignant conversion *in vitro* of epithelial-like cells from rat liver following exposure to chemical carcinogens. Cancer Res. 33: 606 (1973).
79. Montesano, R., Saint Vincent, L., and Tomatis, L. Malignant transformation *in vitro* of rat liver cells by dimethylnitrosamine and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Brit. J. Cancer 28: 215 (1973).
80. Toyoshima, K., et al. *In vitro* malignant transformation of cells derived from rat liver by means of aflatoxin B_1. Gann 61: 557 (1971).
81. Iype, P. T. Studies on chemical carcinogenesis *in vitro* using adult rat liver cells. In: Chemical Carcinogenesis Essays, R. Montesano and L. Tomatis, Eds., IARC, Lyon, 1974, p. 119.
82. Montesano, R., et al. Production of epithelial and mesenchymal tumors with rat liver cells transformed *in vitro*. Int. J. Cancer Res. 16: 550 (1975).
83. Borenfreund, E., et al. Properties and malignant transformation of established rat liver parenchymal cells in culture. J. Nat. Cancer Inst. 55: 375 (1975).
84. Sato, J., et al. Carcinogenesis in tissue culture. VIII. Spontaneous malignant transformation of rat liver cells in long-term culture. Japan. J. Exptl. Med. 38: 105 (1968).
85. Oshiro, Y., Gerschenson, L. E., and DiPaolo, J. A. Carcinomas from rat liver cells transformed spontaneously in culture. Cancer Res. 32: 877 (1972).
86. Rhim, J. S., et al. Induction of hepatocarcinoma *in vivo* with fetal mouse liver cells spontaneously transformed in culture and isolation of a type C RNA virus from the carcinoma cells. Cancer Res. 34: 484 (1974).
87. Schaeffer, W. I., and Polifka, M. D. A diploid rat liver cell culture. III. Characterization of the heterophoid morphological variants which develop with time in culture. Exptl. Cell Res. 95: 167 (1975).
88. Huisingh, J. L., et al. The use of primary rat liver parenchymal cells in evaluating cellular response to toxic metals and carcinogenic polycyclic aromatic hydrocarbons. *In Vitro* 13: 182 (1977).
89. Abernathy, C. O., Lukacs, L., and Zimmerman, H. J. Toxicity of tricyclic antidepressants to isolated rat hepatocytes. Biochem. Pharm. 24: 347 (1975).
90. Zuckerman, A. J., Tsiquaye, K. N., and Fulton, F. Tissue culture of human embryo liver cells and the cytotoxicity of aflatoxin B_1. Brit. J. Exptl. Pathol. 48: 20 (1967).

91. Zuckerman, A. J., et al. The effect of aflatoxins on human embryo liver cells in culture. Brit. J. Exptl. Pathol. 49: 33 (1968).
92. Zuckerman, A. J., Baker, S. F., and Dunkley, L. J. The effects of tetracycline on human liver cells in culture. Brit. J. Exptl. Pathol. 49: 20 (1968).
93. Sullivan, S. F., and Zuckerman, A. J. The effect of heliotrine, a pyrrolizidine alkaloid, on human cells in culture. Brit. J. Exptl. Pathol. 50: 361 (1969).
94. Armstrong, S. J., and Zuckerman, A. J. The effects of lasiocarpine, retrosine, and retronecine pyrrole on human embryo lung and liver cells in culture. Brit. J. Exptl. Pathol. 53: 138 (1972).
95. Armstrong, S. J., and Zuckerman, A. J. Induction of morphological changes in human embryo liver cells by the pyrrolizidine alkaloid lasiocarpine. Brit. J. Exptl. Pathol. 53: 145 (1972).
96. Hadhazy, C., et al. Effets de certains dérivés de la sulfonylureé sur foie de rat en culture organo-typique. II. Examen de l'activite de l'arginase. Pharm. Acta Helv. 46: 539 (1971).
97. Williams, G. M. The direct toxicity of alpha-naphthylisothiocyanate in cell culture. Chem. Biol. Interactions 8: 353 (1974).
98. Benedict, W. F., Gielen, J. E., and Niebert, D. W. Early changes in chromosomal number and structure after treatment of fetal hamster cultures with transforming doses of polycyclic aromatic hydrocarbons. J. Nat. Cancer Inst. 49: 585 (1972).
99. Schwartz, A. The protective effect of estradiol-17β against polycyclic hydrocarbon cytotoxicity. Cancer Res. 33: 2431 (1973).
100. Bauscher, J., and Schaeffer, W. I. A diploid rat liver cell culture. II. Effect of alfatoxin B_1 on ribonucleic acid synthesis. *In Vitro* 9: 294 (1974).